Bacteriocins and Temperate Phage of *Xanthomonas campestris* pv. glycines[1]

Abstract. Sixteen strains of *Xanthomonas campestris* pathovar (pv.) glycines produced bacteriocins (glycinecins) on agar media. Optimal incubation conditions were for 48 h at 20°C. In addition to strains of *X. campestris* pv. glycines, bacteriocins were also inhibitory towards *X. campestris* pv. phaseoli and *X. campestris* pv. vesicatoria. All bacteriocins were susceptible to inactivation by a nonspecific protease and resistant to ribonuclease, but they differed in their sensitivity to trypsin, deoxyribonuclease, and heat treatment. Differential heat and enzyme sensitivities also indicated that some strains of *X. campestris* pv. glycines produce more than one bacteriocin. Attempts to induce bacteriocin production in liquid cultures were unsuccessful. However, temperate bacteriophage were released from cultures of *X. campestris* pv. glycines strains XP175, B83, 17915, and MINN after addition of mitomycin C or nalidixic acid or after exposure to UV light.

Bacteriocins are bactericidal compounds, usually proteinaceous, whose activity is often restricted to bacterial strains that are closely related to the producing bacterium [23]. Many plant pathogenic bacteria, including members of the corynebacteria, erwinias, and pseudomonads, are known to produce proteinaceous bacteriocins [22]. *Agrobacterium radiobacter* strain 84 produces an unusual bacteriocin called Agrocin 84, which is a structural analog of an adenine nucleotide [17].

Bacteriocin production by xanthomonads, however, has received little attention. Hamon et al. [11] claimed bacteriocin production for *X. albilineans, X. betiicola* (sic) and *X. campestris* pathovars juglandis, phaseoli, and vesicatoria, but no data in support of their contention was presented. Liew and Alvarez [14] did not detect bacteriocin production by 85 strains of *X. campestris* pv. campestris using uninduced cultures or after treatment with ultraviolet light or mitomycin C.

The purpose of this study was to determine if strains of the soybean pathogen *X. campestris* pv. glycines are capable of bacteriocin production. This pathogen attacks susceptible soybean leaves, causing bacterial pustule disease, which is characterized by small circular lesions with raised centers [19]. In this paper we demonstrate bacteriocin production by 16 strains of *X. campestris* pv. glycines originating from diverse geographical locations. A preliminary report has been given [7].

[1] Reference to brand or firm names does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

Materials and Methods

Media. Difco nutrient agar (NA) was prepared in water or in 0.05 or 0.1 *M* Na phosphate buffer, pH 7.2. Media (25 ml) was added to each glass culture dish (100 × 15 mm). Difco potatoe dextrose agar (PDA) was prepared in water.

Bacterial strains. The source and geographic origins, where relevant, of the bacterial strains used are listed in Table 1. All *X. campestris* pv. glycines strains were tested by standard procedures [6] and proved to be virulent on soybean cv. Clark.

Bacteriocin production and sensitivity. Standard methods were used for screening for bacteriocin production or sensitivity [9]. Briefly, bacterial suspensions were prepared in sterile water with cells grown on PDA overnight at 28°C. The suspensions were adjusted to OD_{600nm} = 0.01 [approximately 2×10^8 colony-forming units (c.f.u.) per ml, based on plate counts] and six 5-μl droplets of inoculum were spotted onto the surface of buffered or unbuffered NA per dish. Dishes were incubated at 20°C for 48 h, and then the producer cells were killed with chloroform vapors. Sterile water suspensions of bacteria to be used as indicator strains were prepared from overnight cultures grown on NA at

Table 1. Source and geographical origin of bacterial strains

Bacterium	Strain	Source	Country or state of origin
Xanthomonas campestris			
pv. campestris	XC42	UWCC[a]	
	XC43	UWCC	
	XC63	UWCC	
	MPA	R. Gitaitis	
	AC5A	R. Gitaitis	
	AC5⁺A	R. Gitaitis	
	GC	R. Gitaitis	
pv. glycines	1124	NCPPB[b]	Zambia
	1714	NCPPB	Zambia
	1717	NCPPB	Zimbabwe
	XP29	M. P. Starr	Kentucky
	XP144	M. P. Starr (=ATCC[c] 11766)	Indiana
	XP175	M. P. Starr (=NCPPB 554)	Sudan
	XP202	M. P. Starr (=NCPPB 1141)	Zambia
	B83	L. Ferreira	Brazil
	B93	L. Ferreira	Brazil
	B97	L. Ferreira	Brazil
	B99	L. Ferreira	Brazil
	MINN	B. W. Kennedy	Minnesota
	S-9-4	W. F. Fett	Wisconsin
	J3-27-1A	W. F. Fett	Wisconsin
	R12	J. Dunleavy	Iowa
	17915	ATCC	Not known
pv. malvacearum	D	G. R. Lazo	
	G	G. R. Lazo	
	H	G. R. Lazo	
pv. mannihotis	3.25L	L. Sequeira	
pv. pelargonii	L-126	UWCC	
	L-160	UWCC	
pv. phaseoli	BSB	R. Gitaitis	
	27	A. W. Saettler	
	Xp64	UWCC	
pv. pruni	84-22	R. Gitaitis	
	Apricot	E. Civerolo	
	Plum	E. Civerolo	
pv. raphani	70-5	R. E. Stall	
pv. vesicatoria	83-38	R. Gitaitis	
	80-3	R. Gitaitis	
	81-11	R. Gitaitis	
	82-23	R. Gitaitis	
	84-74	R. Gitaitis	
	84-89	R. Gitaitis	
	DG	R. Gitaitis	
pv. vignicola	432	R. Gitaitis	
	80-12	R. Gitaitis	
X. fragaria	APS	B. W. Kennedy	
Bacillus cereus		W. F. Fett	
Erwinia carotovora			
subsp. *atroseptica*	SR8	A. Kelman	
Escherichia coli	11775	ATCC	
Proteus vulgaris	13315	ATCC	
Pseudomonas solanacearum	K60	L. Sequeira	
P. syringae			
pv. glycinea	A-29-2	W. F. Fett	
pv. phaseolicola	3121	P. Lindgren	

[a] UWCC, University of Wisconsin, Department of Plant Pathology Culture Collection.
[b] NCPPB, National Collection of Plant Pathogenic Bacteria, Hatching Green, England.
[c] ATCC, American Type Culture Collection, Rockville, Maryland.

Table 2. Bacteriocin production of *Xanthomonas campestris* pv. glycines

Producer strain	Indicator strain[a]												
	Xcg B93	Xcg B97	Xcg B99	Xcg XP202	Xcg 1714	Xcg XP175	Xcg J3-27-1A	Xcg R12	Xcg XP29	Xcg S-9-4	Xcg XP144	Xcp 27	Xcv 83-38
B83	+[b]	+	+	+	+	+	+	+	+	+	+	+	+
B93	−	−	−	−	−	−	−	−	−	−	+	+	+
B97	−	−	−	−	−	−	−	−	−	−	−	+	+
B99	−	−	−	−	−	−	−	−	−	−	−	+	+
17915	+	+	+	−	+	+	+	+	+	+	+	−	+
XP202	−	−	−	−	−	−	−	−	−	−	+	+	+
MINN	−	+	+	−	−	−	−	−	−	−	+	+	+
1717	−	+	+	−	−	−	−	−	−	−	+	+	+
1714	−	−	−	−	−	−	−	−	−	−	+	−	+
1124	−	+	+	−	−	−	−	−	−	−	+	+	+
XP175	−	−	−	−	−	−	−	−	−	−	+	−	+
J3-27-1A	−	+	−	−	−	−	−	−	−	−	−	−	+
R12	−	−	−	−	−	−	−	−	−	−	+	−	+
XP29	−	+	−	−	−	−	−	−	−	−	−	−	+
S-9-4	−	+	−	−	−	−	−	−	−	−	+	−	+
XP144	−	+	−	−	−	−	−	−	−	−	−	+	+

[a] Xcg, *X. campestris* pv. glycines; Xcp, *X. campestris* pv. phaseoli; Xcv, *X. campestris* pv. vesicatoria.
[b] +, inhibition evident in at least 50% of the assays.

28°C. Suspensions were adjusted to OD_{600nm} = 0.1, and 0.2 ml was added to 9.8 ml of sterile 0.7% (wt/vol) water agar (pH 7.0) maintained at 50°C. The seeded water agar was poured over the surface of the producer plate, and dishes were incubated at 28°C for 24 h. Single plates were prepared for each producer–indicator combination per experiment.

Sensitivity of bacteriocins to heat and enzymes. Inactivation of bacteriocins by heat or by various enzymes was determined by standard procedures [9]. Trypsin (bovine pancreas, Type III, Sigma Chemical Co., St. Louis, Missouri) and protease (Type XIV, Sigma Chemical Co.) were prepared in 0.05 *M* Tris-HCl buffer, pH 7.3, with 0.1 *M* $CaCl_2$ added. Ribonuclease (RNase) (93.7 Kunitz units/mg, bovine pancreas, protease free, Calbiochem-Behring, San Diego, California) was prepared in 0.05 *M* Tris-HCl buffer, pH 8.1. Deoxyribonuclease I (DNase) (bovine pancreas, Type III, Sigma Chemical Co.) was prepared in 0.05 *M* Tris-HCl buffer, pH 8.1 with 10 m*M* $MgSO_4 \cdot 7\ H_2O$ added. All enzyme solutions were prepared at 10 mg/ml. Protease was also prepared at 5 mg/ml.

Producer strains were grown at 20°C for 48 h, killed with chloroform vapors, and enzymes or buffers alone were spotted (10 μl) right next to the producer colonies. After 4 h of incubation at 30°C the producer colonies were overlaid with an appropriate indicator strain as described above. To test for heat inactivation, no enzymes or buffers were spotted, but rather dishes were heated at 75°C for 30 min, and then producer colonies were overlaid with an appropriate indicator strain.

Phage–host range. The temperate phage from strain XP175 was tested for ability to cause plaque formation on bacterial lawns by the soft-agar overlay procedure [1]. Lawns of indicator bacteria were prepared as described above. Six 5-μl droplets of phage preparations were spotted onto freshly prepared lawns per culture dish. Dishes were incubated at 28°C, and lawns were observed for plaque formation for up to 48 h.

Phage morphology. A high-titer stock preparation of the temperate phase present in *X. campestris* pv. glycines strain XP175 was obtained by an agar plate method [4] after three sequential single-plaque isolations to insure the purity of the population. The resultant preparation in NB was subjected to low-speed centrifugation (5090 *g*, 15 min), followed by high-speed centrifugation (100,000 *g*, 4 h). The final pellets were gently taken up in sterile 0.05 *M* Tris-HCl buffer, pH 7.3, containing 20 m*M* $MgSO_4 \cdot 7H_2O$. The titer of this preparation was determined to be 2.9×10^{11} pfu/ml with *X. campestris* pv. glycines strain XP144 as the indicator.

Microaliquots of the preparation were placed on copper grids previously coated with a carbon film and glow discharged. The preparation was allowed to sit on the grid for approximately 1 min and then gently shaken off. A microdrop of 1% phosphotungstic acid, pH 7.3, was placed on the grid, left for 45 s, and then gently shaken off. The edge of the grid was then blotted with a piece of filter paper, and the grids were examined under the electron microscope. A Zeiss EM-10B transmission electron microscope operating at 100 kV was used.

Results

Bacteriocin production and sensitivity. Preliminary experiments using *Xanthomonas campestris* pv. glycines strains 17915 and B83 as producers and strains XP144 and XP175 as indicators indicated that optimal production occurred after 2 days of incubation at 20°C.

All *X. campestris* pv. glycines strains were screened at least two times on unbuffered NA and two times on buffered NA for both their ability to

Table 3. Sensitivity of bacteriocins of *Xanthomonas campestris* pv. glycines to heat and enzymes

Indicator strain	Producer strain	Sensitivity to				
		Trypsin[a]	Protease	RNase	DNase	Heat[b]
X. campestris pv. vesicatoria 83-38	B83, B93, B99, 1124	+	+	−	−	−
	B97, 1714, 1717, 17915 XP29, XP144, XP175 XP202, S-9-4, R12, MINN, J3-27-1A	−	+	−	−	−
X. campestris pv. glycines						
XP144	B83, 1717	−	+	−	−	+
XP175	B83, 17915	+	+	−	+	+

[a] All enzymes tested at 10 mg/ml buffer. Protease also tested at 5 mg/ml.
[b] Heat treatment at 75°C for 30 min.

produce bacteriocins and for their sensitivity to bacteriocin. Results for some producer strain–indicator strain interactions were quite variable, and only those interactions that gave inhibition in at least 50% of the assays are listed as positives (Table 2). Interactions not listed in this table did not result in inhibition, or inhibition occurred in less than 50% of the assays.

Within the strains of *X. campestris* pv. glycines, strains B83 and 17915 gave the most inhibition, and strain XP144 was the most sensitive indicator strain (Table 2). Inhibition zones extended from 1 to 4 mm from the edge of producer colonies.

Strains of *X. campestris* pv. glycines were also screened for ability to inhibit additional pathovars of *X. campestris* (Table 2). *X. campestris* pv. phaseoli strain 27, which is of the fuscans type, and *X. campestris* pv. vesicatoria strain 83-38 were inhibited by most of the *X. campestris* pv. glycines strains. Inhibition zones ranged from 2 to 9 mm from the edge of the producer colonies.

To rule out the possibility that inhibition of *X. campestris* pv. glycines strain XP144, *X. campestris* pv. phaseoli strain 27, and *X. campestris* pv. vesicatoria strain 83-38 was due to a nonspecific sensitivity of these strains to the conditions of the assay, these three strains were tested as indicator strains against *Pseudomonas syringae* pv. glycinea strain A-29-2 and *Escherichia coli* strain 11775 as producers on buffered NA. No inhibition occurred for any of these interactions.

Bacteriophages were ruled out as the inhibitory agents by two methods. In the first method the active agent was tested for transfer from the inhibition zone to a freshly prepared lawn of the indicator strain by use of a sterile wooden toothpick. No inhibitory agent was transferred in this manner. Secondly, agar plugs from the inhibition zones were removed, crushed in a small volume of Difco nutrient broth (NB), and spotted onto a freshly prepared lawn of the indicator strain [3]. Again no transfer of an inhibitor agent occurred.

The heat and enzyme sensitivities of the bacteriocins produced by all *X. campestris* pv. glycines strains inhibitory towards *X. campestris* pv. vesicatoria strain 83-38 were determined. In addition, the bacteriocins produced by strains B83 and 1717, which inhibit strain XP144, and the bacteriocins produced by strains 17915 and B83 active against strain XP175 were also examined. Growth of some of the indicator strains was inhibited by protease alone at 10 mg/ml, so the protease concentration was reduced to 5 mg/ml, which gave no inhibition of bacterial growth. Bacteriocins inhibitory towards *X. campestris* pv. vesicatoria strain 83-38 were all sensitive to the nonspecific protease, but not sensitive to RNase, DNase, and heat (Table 3). Bacteriocin sensitivity to trypsin was variable, with that produced by *X. campestris* pv. glycines strains 1124, B83, B93, and B99 sensitive, while bacteriocins produced by the other 12 strains were resistant. The bacteriocins produced by strains B83 and 1717 that were inhibitory towards strain XP144 were sensitive only to the nonspecific protease and heat. The bacteriocins produced by strains B83 and 17915 that were inhibitory towards strain XP175 were sensitive to trypsin, protease, DNase, and heat. These results indicate that strains 17915 and 1717 produce at least two distinct bacteriocins, while strain B83 produces at least three.

Bacteriocin production was also sought for strains B83, 17915, MINN, and XP175 in NB. No bacteriocins were detected in culture filtrates from uninduced log phase cultures or from cultures treated with mitomycin C (1–5 μg/ml), nalidixic acid (20 μg/ml), or UV light. However, phage were

Table 4. Host range of a temperate bacteriophage of *Xanthomonas campestris* pv. glycines strain XP175

Bacterial strain	Inhibition at[a] RTD	× 10	Bacterial strain	Inhibition at[a] RTD	× 10
Xanthomonas campestris pv. campestris			*X. campestris* pv. pruni		
Xc 42	−	nt[b]	84-22	nt	−
Xc 43	−	nt	Apricot	−	nt
Xc 63	−	nt	Plum	−	nt
MPA	−	nt			
AC5A	−	nt	*P. syringae* pv. phaseolicola		
AC5⁺A	−	nt	3121	−	nt
GC	nt	−			
			P. solanacearum		
X. campestris pv. glycines			K60	−	nt
NCPPB 1124	+	+			
NCPPB 1714	+	+	*Escherichia coli*		
NCPPB 1717	+	+	ATCC 11775	−	nt
ATCC 17915	+	+			
XP29	+	+	*X. campestris* pv. raphani		
XP144	+	+	70-5	nt	−
XP202	+	+			
R12	+	+	*X. campestris* pv. vesicatoria		
B83	+	+	80-3	−	nt
B93	+	+	81-11	−	nt
B97	−	+	82-23	−	nt
B99	+	+	83-38	+	+
MINN	−	+	84-74	+	nt
J3-27-1A	+	+	84-89	+	nt
S-9-4	+	+	DG	+	nt
			X. campestris pv. vignicola		
X. campestris pv. malvacearum			432	+	+
D	nt	−	80-12	−	nt
G	−	nt			
H	−	nt	*X. fragaria*		
			APS	nt	−
X. campestris pv. mannihotis					
3.25L	nt	−	*Pseudomonas syringae* pv. glycinea		
			A-29-2	nt	−
X. campestris pv. pelargonii					
L-126	−	nt			
L-160	−	nt			
X. campestris pv. phaseoli					
Xp64	−	nt			
BSB	nt	−			
27	nt	−			

[a] RTD, routine test dilution (7.6×10^6 pfu/ml); × 10, tenfold higher concentration (7.6×10^7 pfu/ml).
[b] nt = not tested.

detected in culture filtrates of strain XP175 treated at log phase with 1 μg/ml of mitomycin C and of strains B83 and 17915 exposed at log phase to 20 μg/ml of nalidixic acid. Exposure of log-phase cultures of strains XP175 and MINN to UV light also led to the release of phage. Strains XP144 and B83 were used as indicator strains in the assays for phage activity. Plaques were small (0.5–1.0 mm diameter), round with entire margins, and turbid.

Host range and morphology of the temperate phage from *X. campestris* pv. glycines strain XP175. The host range of the temperate phage from strain XP175 was tested by spotting phage preparations containing approximately 7.6×10^6 pfu/ml (the routine test dilution [1] determined with *X. campestris* pv. glycines strain XP144 as indicator) or 7.6×10^7 pfu/ml onto freshly prepared lawns of the indicator bacteria. Results indicated inhibition of all strains of

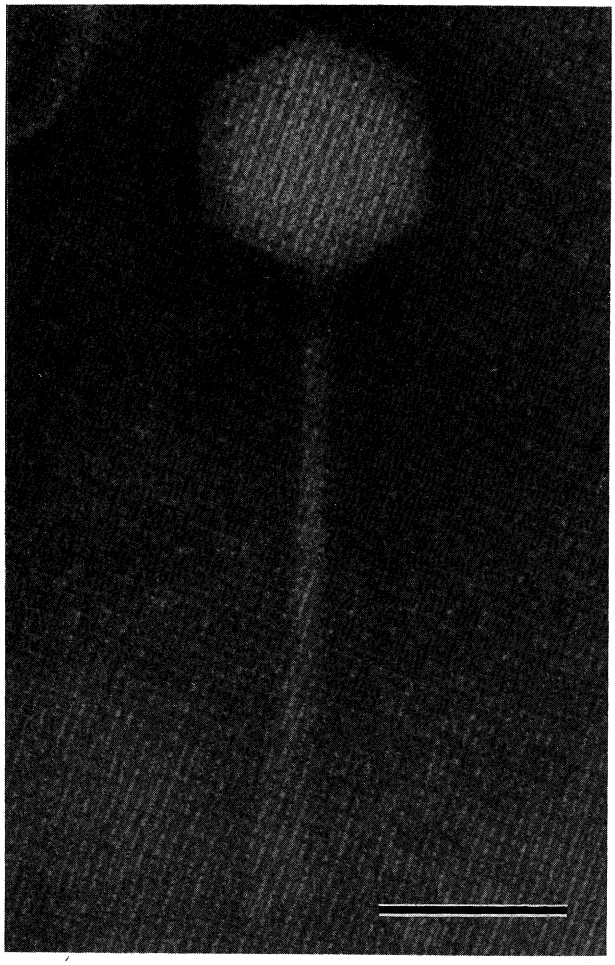

Fig. 1. Electron micrograph of temperate phage in *Xanthomonas campestris* pv. glycines strain XP175. ×500,000; bar = 50 nm.

X. campestris pv. glycines (Table 4). Also inhibited were some but not all strains of *X. campestris* pv. vesicatoria and *X. campestris* pv. vignicola (Table 4). Plaque size and shape were the same as described above.

Under the electron microscope approximately equal numbers of full (Fig. 1) and empty phage particles were observed. Only 1%–2% of the total particles had tails. Heads were isometric and averaged 62 nm in diameter when measured apex to apex. The range was 50–70 nm. The few tails seen were noncontractile, with no tail fibers or collars evident. The apparent widened tail base seen in Fig. 1 is due to the presence of an additional tail of another phage particle. Tails were approximately 175 nm in length.

Discussion

All strains of *X. campestris* pv. glycines were found to be capable of bacteriocin production in vitro on agar media, but bacteriocin production in liquid media could not be induced. We previously proposed the trivial name of glycinecins for these bacteriocins [7], since the trivial name of glycins is already in use for the bacteriocins of *Pseudomonas syringae* pv. glycinea [24]. The proteinaceous nature of the *X. campestris* pv. glycines bacteriocins was indicated by their sensitivity to a nonspecific protease preparation.

That the bacteriocins produced by *X. campestris* pv. glycines were not all identical was indicated by their differential sensitivity to heat and enzyme treatments. In addition, differential sensitivities to heat, trypsin, and DNase indicate that *X. campestris* pv. glycines strains 17915, B83, and 1717 produce more than a single bacteriocin. Production of more than one bacteriocin by a single bacterial strain has also been reported for *Corynebacterium* species [9].

One of the bacteriocins produced by *X. campestris* pv. glycines strain 17915 and strain B83 was sensitive to DNase. Bacteriocins containing DNA are unusual but not without precedent. The so-called "killer particles" of *Bacillus* appear under the electron microscope as complete, phage-like particles and contain DNA [2]. *Bacillus* "killer particles" are thermolabile, as are the DNase-sensitive bacteriocins of strains 17915 and B83. DNA-containing "killer particles" have also been reported for *Agrobacterium tumefaciens* and *A. radiobacter* [21]. Low-molecular-weight bacteriocins produced by *Rhizobium trifolii* are also reported to be sensitive to DNase [18].

Optimal bacteriocin production by *X. campestris* pv. glycines on agar media occurred at 20°C, well below the optimal in vitro growth temperature of 30°C for this organism [19]. Maximum bacteriocin production at a temperature lower than the optimal growth temperature has also been reported for phytopathogenic corynebacteria [9] and pseudomonads [24].

Bacteriocins produced by *X. campestris* pv. glycines strains 1124, B83, B93, and B99, which inhibit *X. campestris* pv. vesicatoria strain 83-38, are trypsin sensitive and thermostable; this indicates that they may be of low molecular weight, while the bacteriocins produced by strains B83 and 1717, which inhibit strain XP144, are trypsin resis-

tant and thermolabile, which indicates that they may be of high molecular weight on the basis of findings for other bacteriocins [2].

All of the strains of *X. campestris* pv. glycines used in this study harbor from one to three plasmids [10]. Naturally occurring variants of *X. campestris* pv. glycines strain B83, which do not produce bacteriocin on NA, do not show detectable changes in plasmid content and size (M. J. Haas and W. F. Fett, unpublished). Thus, it appears that bacteriocin production by *X. campestris* pv. glycines is not encoded by genes carried on a plasmid. However, the possible involvement of megaplasmids has not been ruled out.

In contrast to the limited number of studies on bacteriocin production by xanthomonads, there have been numerous studies on bacteriophage capable of infecting xanthomonads [16, 22]. Xanthomonad phages isolated from diseased plant tissues usually have a narrow host range restricted to the xanthomonads [8, 20]. Numerous virulent phages of xanthomonads have been reported, while temperate phages have been obtained only from *X. campestris* pv. glycines [15], *X. campestris* pv. malvacearum [12], *X. campestris* pv. pruni [5], and *X. campestris* pv. oryzae [25]. Xanthomonad phage morphologies range from filamentous [13] to polyhedral, with a tail and contractile sheath [14].

The morphology of the temperate phage from *X. campestris* pv. glycines strain XP175 falls into Group B of Bradley's classification scheme [2], namely, phage with polygonal heads and long, noncontractile tails. Group B phage normally have double-stranded DNA. Numic [15] previously detected a temperate phage (Xpg 10) from *X. campestris* pv. glycines strains X55 that was inducible by UV light treatment. This DNA-containing temperate phage had a polygonal head (60 × 60 mμ), a long, noncontractile tail (143 × 11 mμ), and a thermal inactivation point of 68°C. The phage was proposed to be specific for *X. campestris* pv. glycines, but only a limited number of *X. campestris* pathovars were tested in host range studies [15].

In addition to *X. campestris* pv. glycines strains XP175 and X55, strains B83, 17915, and MINN also appear to carry a temperate phage. Determination of the relationships among these temperate phages awaits further study.

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